Increased lung neutrophil apoptosis and inflammation resolution in

nonresponding pneumonia

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Abstract

Question: Neutrophil activation state and its relationship with an inflammatory environment in community-acquired pneumonia (CAP) remain insufficiently elucidated. We aimed to evaluate the neutrophil apoptosis and cytokine pattern in CAP patients after 72 hours of treatment, and their impact on infection resolution.

Methods: Apoptosis of blood and bronchoalveolar lavage (BAL) neutrophils was measured in nonresponding CAP (NCAP), in responding CAP (blood only) and in patients without infection (control). Pro-inflammatory (IL-6, IL-8) and anti-inflammatory (IL-10) cytokines were measured. Main outcomes were clinical stability and days of hospitalization.

Results: Basal neutrophil apoptosis was higher in BAL and blood of NCAP whereas spontaneous apoptosis (after 24h culture) was lower. Cytokines in NCAP were higher than in responding CAP and control: IL-6 was increased in BAL and blood, IL-8 in BAL and IL-10 in blood. An increased basal apoptosis ($\geq$20%) in BAL of NCAP was associated with lower systemic IL-10 (p<0.01), earlier clinical stability (p=0.05) and shorter hospital stay (p=0.02). A significant correlation was found for systemic IL-6 and IL-10 with days to reach stability and length of stay.

Answer the question: After 72 hours of treatment, an increased basal alveolar neutrophil apoptosis might contribute to downregulate inflammation and to faster clinical stability.

199 words

Key words: bronchoalveolar lavage; cytokines; length of stay; neutrophil apoptosis; nonresponding community-acquired pneumonia.

Introduction

Community-acquired pneumonia (CAP) has an incidence of 5/1,000 adults [1] and its mortality ranges between 5% and 15% of hospitalised patients. An adequate host response facilitates microbial killing while limiting excessive inflammation and tissue damage.[2] However, approximately 10% of CAP patients develop nonresponding pneumonia (NCAP), which leads to delayed resolution and poor outcome.[3]

In CAP, blood activated neutrophils migrate and accumulate into the lungs, ingest and kill bacteria and, finally, undergo apoptosis. Macrophages remove apoptotic neutrophils, precluding subsequent release of inflammatory cytokines and promoting resolution of inflammation.[4, 5] Although neutrophils are usually short-lived immune cells, their lifespan prolongation is critical in their efficacy. However, as neutrophils' lytic enzymes can also induce organ damage, their activation and survival must be tightly regulated.[5-9]

In NCAP a persistent lung and systemic inflammation has been reported.[10-12] However, the state of neutrophil activation remains to be elucidated. We hypothesised that the persistence of activated neutrophils in lung of NCAP might be contributing to the maintenance of inflammation and to a delayed resolution.

We aimed to evaluate the neutrophil apoptosis and the cytokine pattern (IL-6, IL-8 and IL-10) both in lung and in blood in hospitalised patients with NCAP. Secondarily, we investigated their impact on the clinical parameters of stability and length of stay.

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Methods and Materials

Study design

A prospective study was performed in admitted patients with CAP. Pneumonia was defined as the presence of a new infiltrate with concordant symptoms and laboratory results (fever $\geq 38^{\circ}C$, purulent sputum, leukocyte count $\geq 12.000/mm^3$). Exclusion criteria were admission in the previous 15 days and immunosuppressive treatment.

The inclusion criteria in NCAP group: to fulfil clinical conditions of NCAP after 72 hours of treatment (see definition below) and performance of bronchoscopy indicated by the physician in charge. Patients were included in the study the day when they underwent bronchoscopy.

Two control groups matched by age (±10 years) and comorbid conditions were included: 1. CAP group comprised patients with CAP who reached clinical stability (definition below) 2. Control group: Patients without infection referred to bronchoscopy for peripheral lung nodules or minor haemoptysis.

We collected data on age, gender, smoking habits, comorbidities, clinical signs and symptoms, Pneumonia Severity Index (PSI),[13] chest radiograph, biochemical analyses, microbiological findings, and previous and current antibiotic treatment.

The study was approved by the Ethics Committee of our Center and written informed consent was obtained. No BAL fluid was obtained in the CAP group because the Ethics Committee would not approve bronchoscopy in patients with adequate response.

Definitions

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NCAP[12] was defined as persistence of a high temperature (≥38 ºC) and/or clinical symptoms after 72 hours of antibiotic treatment, chest-X ray progression of pneumonia (>50% increase of infiltrates along with persistence of high temperature and/or clinical symptoms), empyema, septic shock and/or the need for mechanical ventilation.

The CAP group comprised patients who fulfilled clinical stability criteria:[14, 15] Stability was defined as the achievement of all the following parameters: temperature ≤37.2 ºC, heart rate ≤100 beats/min, respiratory rate ≤24 breaths/min, systolic blood pressure ≥90 mm Hg, and oxygen saturation ≥90% or Pa O_2 ≥60 mm Hg without supplemental oxygen.

Length of stay (LOS) was calculated as the number of days from admission until discharge.

Obtaining and processing of bronchoalveolar lavage

BAL was performed according to recommended guidelines.[16] In the NCAP group, BAL was performed in the involved lobe and in those patients with diffuse pulmonary infiltrates or in the control group, in the middle lobe or lingula. Five aliquots of sterile saline solution were instilled and immediately aspirated. The first aliquot (20 mL) was discarded. The remaining four aliquots (30 mL each) were pooled together in a single siliconised sterile glass: half of these four aliquots were sent to a microbiology laboratory and the other half to a biochemistry laboratory. The mean volume of BAL fluid obtained was 39±3 mL.

Neutrophil purification from bronchoalveolar lavage samples

The BAL samples were immediately pelleted by centrifugation at 353xg for 5 min at 4ºC. The supernatant volume was measured and frozen. The pellet was resuspended with culture medium (RPMI 1640 w/o phenol red, Invitrogen, Carlsbad,

CA), passed through two sheets of gauze, and centrifuged at 353xg for 5 min at 4ºC. The cell pellet was washed twice with PBS and resuspended with hypotonic solution (NH$_4$Cl 155 mM, NaHCO$_3$ 2,46 mM, EDTA4H$_2$O 3,72 mM pH 7,4) for 5 min on ice. After centrifugation at 304xg for 5 min at 4ºC, the pellet was resuspended in PBS. The neutrophils' amount and viability were determined using a trypan blue exclusion method[17, 18]. For light microscopies, cytospin slides were stained by the Panoptic method (Panreac Quimica SA, Barcelona, Spain).

Preparation of blood samples

In NCAP, blood samples were obtained before bronchoscopy, and in the CAP group, after reaching stability. Plasma aliquots were withdrawn before neutrophils isolation for cytokines determination by ELISA. The isolation of neutrophils was developed as previously described.[19] The pellet was resuspended with a hypotonic solution for 5 min on ice, and spun down with PBS 304xg for 5 min to pellet the neutrophils (similar to BAL samples). Cells were resuspended in PBS, and counted.

Detection of apoptosis by flow cytometry

Neutrophils were stained with Annexin V detection kit (R&D, TACS^{TM} Annexin V-FITC, MN, USA) following the manufacturer's instructions. Samples were analysed using an EPICS XL (Beckman-Coulter, Hialea, FL, U.S.A.).[20] Neutrophils were gated according to their forward and side scatter characteristics with a minimum of 10.000 gated events from each sample. Controls were included to set-up compensation with no stained cells and cells stained with Annexin V or PI alone. Apoptotic cells were distinguished from normal and necrotic cells by labelling with annexin V and exclusion of propidium iodide. Each subpopulation was expressed as a percentage of the total neutrophil population.

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In addition, staining with CD16 FITC (IOTest, Beckman Coulter) in separate aliquots of BAL and blood samples was applied to detect the positive neutrophil population.

For detection of apoptosis increments (spontaneous apoptosis) after 24 hours of in vitro culture, neutrophils were seeded in RPMI supplemented with 10% FBS, L-glutamine and antibiotics (penicillin and gentaminicin, Invitrogen) and cultured at 37ºC for 24 hours.

Measurement of cytokines IL-6, IL-8, IL-10 in BAL and plasma.

The determination of IL-6, IL-8, and IL-10 was performed with a commercial enzymoimmunoassay technique (Pharmingen, BD Biociencias, Madrid, Spain) following the instructions of the manufacturer. The limits of detection in pg/mL were: 4.7 for IL-6, 3.1 for IL-8, and 7.8 for IL-10.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 and GraphPad software (San Diego, CA). The chi-square test was used for categorical variables, and the Mann-Whitney U test for continuous variables. Correlation was analysed using Spearman´s rho correlation analysis. Data are shown as mean±SEM unless otherwise indicated. Cytokine levels and LOS are shown in medians and interquartil ranges (IQR). A value of $p < 0.05$ was considered statistically significant. The endpoint variables were analysed with regard to the basal neutrophil apoptosis in BAL stratified as high ($\geq 20\%$, 75 percentile of basal apoptosis) or low ($< 20\%$) apoptosis rates.

Results

General characteristics of groups

Sixty patients (29 NCAP, 15 CAP and 16 controls without infection) were recruited. Six NCAP patients were excluded due to an alternative diagnosis: 1 tuberculosis, 3 lung cancer, 1 bronchiolitis obliterans and organizing pneumonia, and 1 invasive pulmonary aspergillosis, thus leaving 23 in the study group. Features of nonresponding pneumonia were: 11 persistent fever and clinical worsening, 9 progression of infiltrates in chest radiograph (two with pleural effusion), and 3 respiratory insufficiency requiring invasive ventilation; one of these patients died. The control group without infection included 16 adults, 7 minor haemoptysis and 9 peripheral lung nodules, whose diagnosis ruled out infection and whose comorbidities, sex and age were similar to those in the NCAP group and CAP group (Table 1).

The median interval since admission to the day in which samples were taken in the NCAP group was 6 days (4-8), and 5 days (4-5) in the CAP group.

Etiologic diagnosis in NCAP group was established in 14 cases (60.9%): 7 *S pneumoniae*, 1 *E Coli*, 2 methicillin resistant *S aureus (MRSA)*, 1 *P aeruginosa* and 5 mixed etiology (3 *S pneumoniae* and other microorganisms, 2 *MRSA* and other). In BAL fluid, the following microorganisms were isolated: 1 *S. pneumoniae*, 2 *Streptococus spp*, 1 *Enterococcus spp*, 1 *P. aeruginosa*, 2 *MRSA*, 1 *A. baumanii*, 10 potentially non pathogenic microorganisms, and none in 3 cases. Etiology in CAP group was: 4 *S pneumoniae* (27%),1 *P. aeruginosa* (7%), and unidentified in 10 cases.

Apoptosis and cytokine results in BAL and blood

Leukocyte counts in blood (Table 1) and BAL fluid were significantly higher in the NCAP group. Apoptosis values from flow cytometry (Figure 1a) were compared to data obtained by judging the nuclear morphology by light microscopy (Figure 1b) and similar results were obtained.

The basal apoptosis in BAL (Figure 2a) was higher (13±3%) in NCAP compared to control (6±3%) though significance was not reached; no data were available for CAP (as described in material and methods). A similar pattern in blood was observed in NCAP compared to control and CAP (15±4 %, 4±1% and 6±2% respectively). No significant correlation was found between apoptotic neutrophils in BAL and in blood in the NCAP group. Spontaneous apoptosis after 24 hours of culture was studied in a subset of patients (n=5) to observe neutrophil apoptosis *in vitro* (Figure 2b). Spontaneous apoptosis was lower in blood in NCAP (compared to control and CAP), $p<0.05$. The same pattern appeared in BAL (compared to control, no data for CAP) though significance was not reached.

The median levels of systemic cytokines are depicted in Figure 3. IL-6 was significantly higher in BAL of the NCAP (compared to control), and in blood (compared to control and a trend compared to CAP). IL-8 was higher in BAL in NCAP (compared to control) although no differences were found in blood (compared to control and CAP). On the contrary, IL-10 showed significant higher levels in blood (compared to control and CAP) without differences in BAL (compared to control).

In the NCAP group, cytokine levels in BAL compared with blood showed significant higher IL-8 BAL levels, 4358 pg/mL (IQR: 868-12117) vs. 38 (24-78), and significant higher IL-10 in blood, 22 (9-60) vs. 3 (2-9) pg/mL, whereas no differences were found concerning IL-6.

In the NCAP group, a statistically significant positive correlation was found between IL-6 in blood and BAL fluid (r:0.7, p=0.01). IL-6 levels in BAL were also found significantly correlated to IL-10 levels in blood (r:0.7, p=0.02). A significant correlation of the percentage and absolute count of neutrophils in BAL was found with IL-8 in BAL (r:0.6, p=0.01 and r:0.7, p<0.01, respectively).

Clinical resolution and outcome

One of the NCAP patients died. The median number of days to reach clinical stability was 12 (IQR:7-18), and the median LOS was 20 (12-34). Basal high neutrophil apoptosis in BAL (≥20%) showed a significant negative correlation with LOS (r:-0.5, p=0.02) and with days needed to reach clinical stability (r:-0.4, p=0.05). Patients with high apoptosis in BAL had shorter LOS (median 10 vs. 21 days) and needed fewer days for stability (median 7 vs. 13 days). Neutrophil apoptosis in blood did not correlate either with clinical stability or with LOS (r:-0.2, p=0.4 and r:-0.1, p=0.7 respectively).

Cytokine levels in NCAP stratified according to high (≥20%) or low (<20%) basal BAL neutrophil apoptosis are depicted in Figure 4. Systemic IL-10 was significantly lower in those with high apoptosis. A trend to lower IL-6 and IL 8 was found in those with high apoptosis both in BAL and blood. IL-6 e IL-10 levels in blood showed a positive significant correlation with the number of days to reach clinical stability (r:0.7, p <0.01; r:0.7, p<0.01 respectively), and IL-10 with LOS (r:0.7, p=0.02). In the CAP group, a significant positive correlation was found for blood IL-6 and IL-10 with number of days to reach clinical stability (r:0.4, p=0.02 and r:0.7, p<0.001 respectively), and with LOS (r:0.4, p=0.03 y r:0.7, p<0.001 respectively).

Discussion

The most important findings of this study are: (1) Systemic basal neutrophil apoptosis was significantly higher in the NCAP group than in responding CAP and control, while spontaneous apoptosis at 24 hours was lower. (2) The inflammatory cytokine pattern showed higher pro-inflammatory IL-6 and IL-8 in BAL, and in blood higher IL-6 along with higher IL-10. (3) Patients with NCAP who had increased basal apoptosis in BAL ($\geq$20%) reached clinical stability earlier and their LOS was shorter. (4) Low basal alveolar apoptosis was associated with higher IL-10 in blood and a trend for higher IL-8 and IL-6 in BAL and blood.

The apoptosis of neutrophils after killing microorganisms is considered essential to downregulate inflammation while precluding the release of their cytotoxic components. The relevance of high initial and persistent inflammation in CAP has been thoroughly studied. Initial raised inflammatory cytokines in NCAP and at 72 hours have been associated with slower clinical stability and poorer outcome.[12, 21-23] As expected, our findings showed an increase in cytokine levels and alveolar neutrophils, which reflects an ongoing inflammation along with certain degree of apoptosis. In fact, we found higher basal neutrophil apoptosis in BAL (12%) than Droemann et al[24] (1%), probably because they included patients in an earlier stage. Interestingly, we also found a slightly higher neutrophil apoptosis in blood reflecting the functional continuum of the infection.

Although absolute basal apoptosis was increased, neutrophils' survival was also prolonged, that is, their functional longevity is enhanced. Spontaneous apoptosis at 24 hours was higher in controls than in NCAP, as reported elsewhere.[4] This finding illustrates the neutrophil fate during infection, especially because BAL neutrophils were

not totally purified from the rest of BAL cells, and cytokine released by co-cultured cells would reflect the *in vivo* inflammation process. Interestingly, in patients with clinical stability, both basal and spontaneous apoptosis mimics the pattern of controls without infection. The lower systemic basal apoptosis in CAP represents a phase where leukocytes are declining, such as it corresponds to a resolving infection with decreasing cytokines (IL-6 and IL-10). These results indicate that a different neutrophil apoptosis kinetic is a feature of NCAP patients.

Activation of neutrophils is necessary to clear infection, as well as their apoptosis to adequately resolve inflammation.[25] During the initial phases of infection, cytokines may enhance neutrophil activation, mainly IL-8 prime neutrophils and prolong their lifespan.[26] We have confirmed the higher pro-inflammatory cytokine levels of IL-6 and IL-8 in BAL fluid after 72 hours of treatment, as expected in NCAP patients. Interestingly, the predominant inflammatory cytokines in the pulmonary and systemic compartments revealed different patterns: higher IL-8 in BAL, higher IL-10 in blood and similar IL-6 levels in both compartments. Schütte et al have also reported similar findings with IL-8 and IL-6 in patients with severe pneumonia.[27] The higher IL-8 levels in BAL compared to blood highlight the active phase of infection and the upregulation of neutrophil. In fact, a positive correlation between IL-8 and the absolute count of neutrophils in BAL was confirmed. Concerning IL-6, we found a positive correlation between levels in BAL and in blood, which indicates that an inflammatory process originated in the lung is continued in the systemic compartment. On the contrary, IL-10 levels in blood indicate a predominant anti-inflammatory pattern. In fact, significant systemic lower IL-10 and a trend for lower IL-6 was found in patients with clinical stability. Taking together all these results, a different speed of inflammatory resolution is suggested: slower in lung than systemically. Moreover, we

found a correlation between systemic inflammatory resolution and clinical resolution; that is among systemic IL-6 and IL-10 and stability and LOS.

The interaction between alveolar neutrophils, cytokines and adequate restoration of homeostasis is far to be totally understood. Our results indicate that, when alveolar apoptosis was low in BAL, systemic IL-10 and IL-8 in BAL fluid were raised. This association may have several explanations. First, a reduced apoptosis may reflect an ongoing inflammation with the potential for prolonging the tissue damage. Second, bacterial load or the pathogens themselves might alter apoptosis thus evading their efficient killing.[26] Third, an exuberant cytokine environment might delay apoptosis, as reported in acute lung injury, where is associated with a dysregulation of apoptosis.[28] In sepsis, a delayed apoptosis due to prolonged activation of nuclear factor kB causes neutrophils to remain active.[29,30] Enhancement of neutrophil apoptosis, on the contrary, has been associated to less lung damage and mortality.[31] In an animal model localised apoptosis was found to be a feature in resolution of pneumonia.[32]

Clinical relevance of alveolar apoptosis was proved when analyzing resolution of infectious parameters. We found that a higher alveolar apoptosis was significantly associated with faster clinical stability and shorter LOS. A similar significant correlation was found between systemic levels, both in NCAP and responding CAP, of cytokines, clinical stability and LOS.

To our knowledge, this is the first study aimed to investigate the role of neutrophil apoptosis both in lung and systemically in NCAP. As potential limitations we have to point out, a possible beta error in statistical analyses due to the limited number of cases included and the lack of BAL in the responding CAP group.

In summary, basal apoptosis in NCAP is high both in lung and in blood while spontaneous apoptosis at 24 hours is lower than in responding CAP. High IL-8 levels in BAL fluid were associated with accumulation of neutrophils in lung. Systemic inflammation with raised IL-6 and IL-10 is associated with a delayed clinical stability and LOS. In NCAP, a greater basal alveolar neutrophil apoptosis was associated with earlier clinical stability and lower LOS, which suggests that neutrophil apoptosis precludes persistent local inflammation thereby contributing to a faster recovery of normal homeostasis.

Acknowledgments

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Table 1: Characteristics of NCAP and control groups

	NCAP (n 23)	CAP (n 15)	Control (n 16)	p value*
Age, years	61±3	66±4	60±2	NS
Male/Female	16/7	12/3	14/2	NS
Smoker/non-smoker	6/17	13/2	9/7	NS
Comorbidities, n(%)				
-Heart disease	6 (26)	3(20)	2(12)	NS
-COPD	7 (30)	7(46)	5(31)	NS
-Chronic renal disease	2 (9)	0	1(6)	NS
-Cerebrovascular disease	3 (13)	3(20)	1(6)	NS
PSI score	90±6	102±7	0	
Blood leukocyte count (cells/mm^3).	12,752±1,268	9,877±1,065	8,217±730	0.02
-PMN %	82±11	76±3	62±4	0.001
BAL fluid Total cells/ mm^3	7.5±2.2		0.5±0.2	p=0.01
-PMN%	38±4.5		8±3	p<0.001

Abbreviations: NCAP, nonresponding pneumonia; COPD, chronic obstructive pulmonary disease; PSI, pneumonia severity index (FINE-score); PMN, polymorphonuclear neutrophils

Results are expressed as mean±SEM

*Mann-Whitney U test and chi-square test for continuous and categorical variables, respectively.

Figure legends

Figure1: A) Flow cytometry of one representative BAL sample. Neutrophils were gated according to their forward (FS) and side scatter (SS) characteristics (left histogram). To confirm neutrophil population of gated area, cells were stained with CD16 antibody (FITC), as shown in the middle histogram. Right histogram: gated area, containing neutrophils. Horizontal axis represents intensity of staining for Annexin V (FITC, logarithmic scale) and vertical axis intensity of staining for Propidium iodide (PI, logarithmic scale). B) Light microscopy of BAL neutrophils in cytocentrifuge slides.
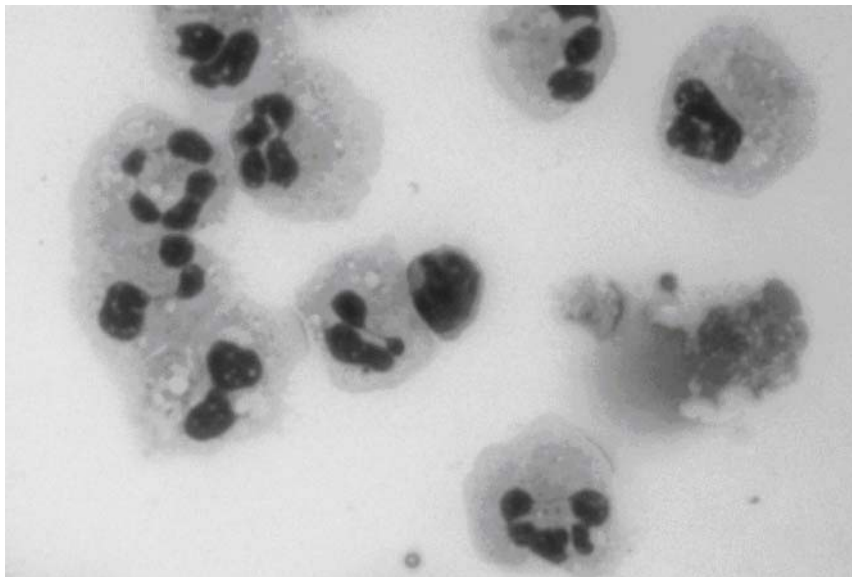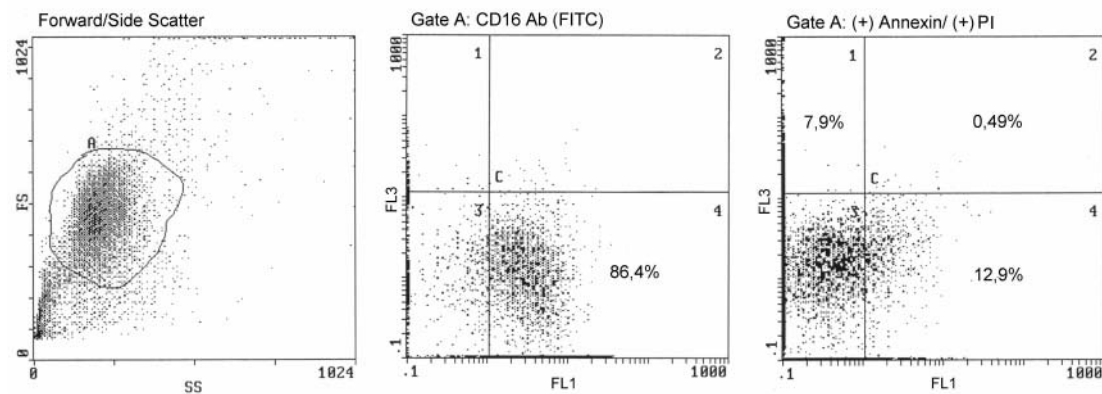
Figure 2: Apoptosis of neutrophils in bronchoalveolar lavage (BAL) of NCAP and control (2a) and peripheral blood of NCAP, control and CAP (2b). Apoptosis was determined after neutrophils purification (basal apoptosis) and after *in vitro* culture for 24 hours (spontaneous apoptosis). *$p<0.05$ NCAP vs Control, # $p<0.05$ NCAP vs CAP.
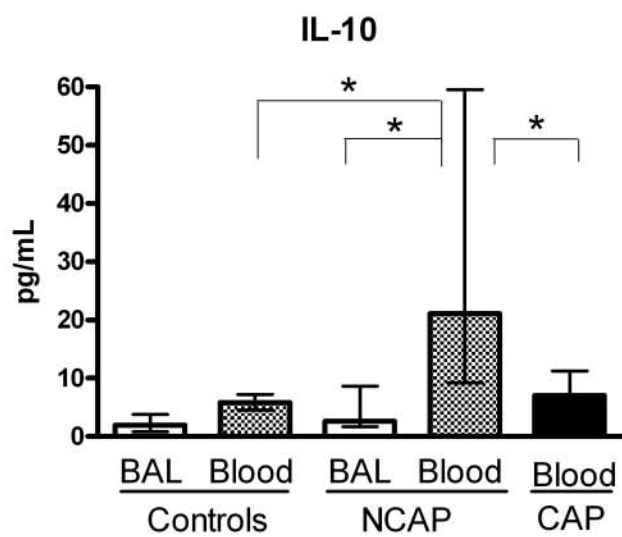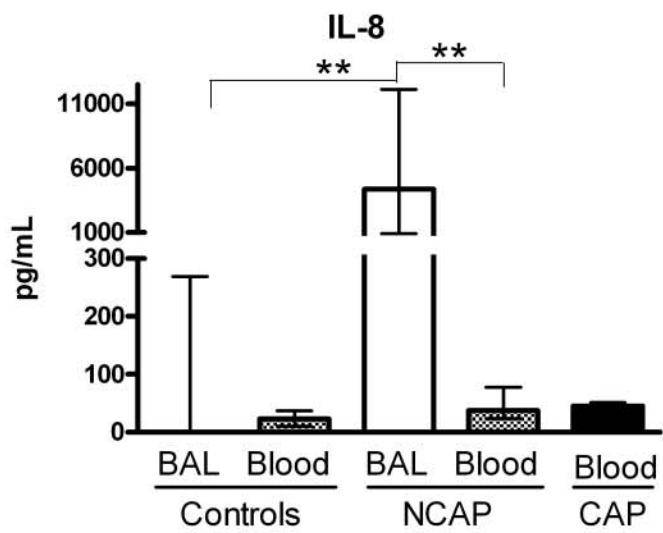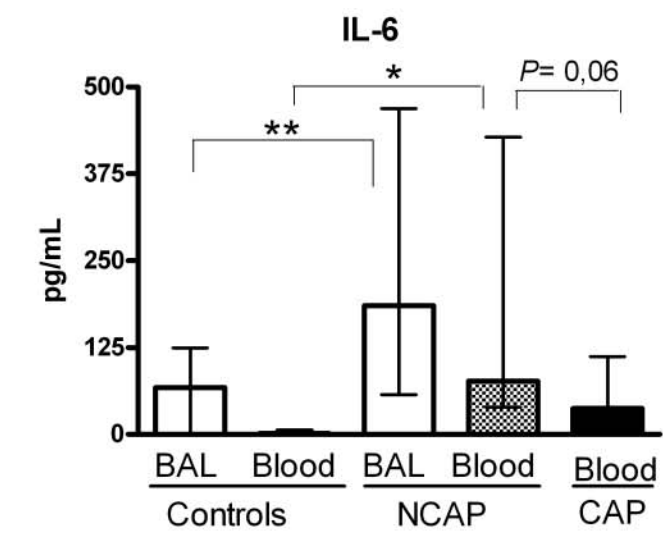
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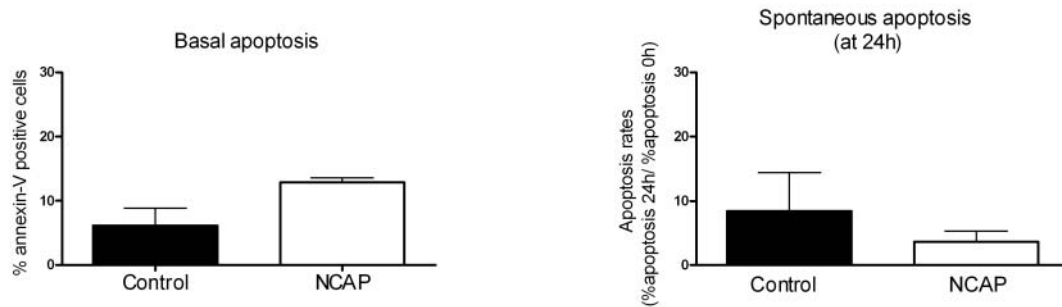
2b, apoptosis in blood neutrophils
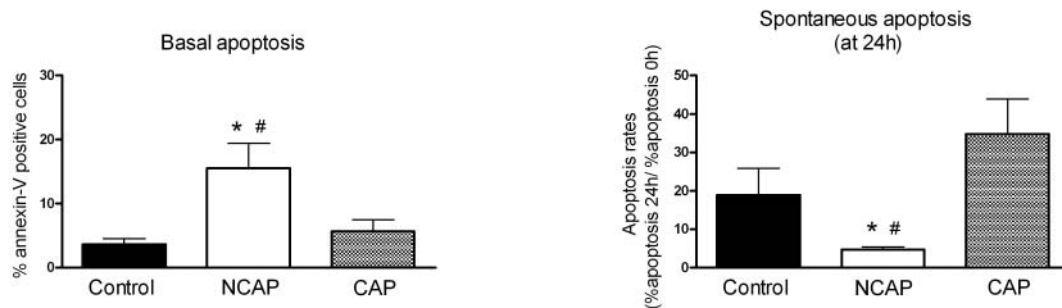
Figure 3: Cytokine levels from NCAP, CAP and control group in bronchoalveolar lavage (BAL) and peripheral blood.* $p< 0.05$, ** $p< 0.01$.

IL-6

IL-8

IL-10

Figure 4: Cytokine levels in bronchoalveolar lavage (BAL) and blood of NCAP patients classified as low (<20%) or high BAL apoptosis (≥20%). * *p< 0.05*

IL-6

IL-8

IL-10